Amendments to the Specification:

Please replace the title with the following amended title:

~~NOVEL VECTORS IN AVIAN TRANSGENESIS~~ EGGS CONTAINING EXOGENOUS PROTEIN

Please replace the paragraph on page 2, beginning on line 14, with the following amended paragraph:

--The avian reproductive system, including that of the chicken, is well described. The egg white of the hen consists of several layers which are secreted upon the yolk during its passage through the oviduct. The production of an egg begins with formation of the large yolk in the ovary of the hen. The unfertilized oocyte is ~~then~~ positioned on top of the yolk sac. Upon ovulation or release of the yolk from the ovary, the oocyte passes into the infundibulum of the oviduct where it is fertilized if sperm are present. It then moves into the magnum of the oviduct which is lined with tubular gland cells. These cells secrete the egg-white proteins, including ovalbumin, lysozyme, ovomucoid, conalbumin, and ovomucin, into the lumen of the magnum where they are deposited onto the avian embryo and yolk.--

Please replace the paragraph on page 6, beginning on line 22, with the following amended paragraph:

--~~Lui, *Poult. Sci.* 68:999-1010 (1995), used a~~ A targeting vector containing flanking DNA sequences of the *vitellogenin* gene has been used to delete part of the resident gene in chicken blastodermal cells in culture. However, it has not been demonstrated that these cells can contribute to the germ line and thus produce a transgenic embryo. In addition, this method is not useful when the deleted gene is required for survival or proper development of the organism or cell type.--

Please replace the paragraph on page 12, beginning on line 11, with the following amended paragraph:

-- Fig. 2(e) illustrates a method of amplifying an exogenous gene for insertion into the vectors of 2(a) and 2(b), wherein the nucleic acid sequence encoding the lysozyme signal peptide comprises the nucleotide sequence of SEQ ID NO: 1.

Please replace the paragraph on page 19, beginning on line 13, with the following amended paragraph:

--Figs. 1(a) and 1(b) illustrate examples of *ovalbumin* promoter expression vectors. Gene *X* is a coding sequence which encodes an exogenous protein. Bent arrows indicate the transcriptional start sites. In one example, the vector contains 1.4 kb of the 5' flanking region of the *ovalbumin* gene (Fig. 1(a)). The sequence of the "-1.4kb promoter" of Fig. 1(a) corresponds to the sequence starting from approximately 1.4kb upstream (-1.4kb) of the *ovalbumin* transcription start site and extending approximately 9 ~~residues~~ nucleotides into the 5'untranslated region of the *ovalbumin* gene. The approximately 1.4 kb-long segment harbors two critical regulatory elements, the steroid-dependent regulatory element (SDRE) and the negative regulatory element (NRE). The NRE is so named because it contains several negative regulatory elements which block the gene's expression in the absence of hormone. A shorter 0.88 kb segment also contains both elements. In another example, the vector contains approximately 7.4 kb of the 5' flanking region of the *ovalbumin* gene and harbors two additional elements (HS-III and HS-IV), one of which is known to contain a functional region enabling induction of the gene by estrogen (Fig. 1(b)). A shorter 6 kb segment also contains all four elements and could optionally be used in the present invention--

Please replace the paragraph on page 23, beginning on line 26, with the following amended paragraph:

-- Fig. 3(a) shows a schematic of the replication-deficient avian leukosis virus (ALV)-based vector pNLB, a vector which is suitable for use in this embodiment of the invention. In the

pNLB vector, most of the ALV genome is replaced by the neomycin resistance gene (*Neo*) and the *lacZ* gene, which encodes ~~b-galactosidase~~ β-galactosidase. Fig. 3(b) shows the vector pNLB-CMV-BL, in which *lacZ* has been replaced by the CMV promoter and the *β-lactamase* coding sequence (*β-La* or *BL*). Construction of the vector is reported in the specific example, Example 1, below. *β-lactamase* is expressed from the CMV promoter and utilizes a poly adenylation signal (pA) in the 3' long terminal repeat (LTR). β-Lactamase has a natural signal peptide; thus, it is found in blood and in egg white.--

Please replace the paragraph on page 24, beginning on line 6, with the following amended paragraph:

-- Avian embryos have been successfully transduced with pNLB-CMV-BL transduction particles (see specific examples, Example 2 and 3, below). The egg whites of eggs from the resulting stably transduced hens were found to contain up to ~~20 mg~~ 70 μg of secreted, active ß-lactamase per egg (see specific examples, Example 4 and 5, below). --

Please replace the paragraph on page 24, beginning on line 21, with the following amended paragraph:

-- Optionally, site-specific recombination systems, such as the Cre-*lox*P or FLP-FRT systems, are utilized to implement the magnum-specific activation of an engineered constitutive promoter. In one embodiment, the first transgene contains an FRT-bounded blocking sequence which blocks expression of the first coding sequence in the absence of ~~FTP, and the second coding sequence encodes FTP~~ FLP, and the second coding sequence encodes FLP. In another embodiment, the first transgene contains a *lox*P-bounded blocking sequence which blocks expression of the first coding sequence in the absence of the Cre enzyme, and the second coding sequence encodes Cre. The *lox*P-bounded blocking sequence may be positioned in the 5' untranslated region of the first coding sequence and the *lox*P-bounded sequence may optionally contain an open reading frame.--

Please replace the paragraph on page 25, beginning on line 1, with the following amended paragraph:

-- For instance, in one embodiment of the invention, magnum-specific expression is conferred on a constitutive transgene, by linking a cytomegalovirus (CMV) promoter to the ~~coding sequence of the protein to be secreted (CDS)~~ coding sequences (CDS) of the protein to be secreted (Figs. 6(a) and 6(b)). The 5' untranslated region (UTR) of the coding sequence contains a *lox*P-bounded blocking sequence. The *lox*P-bounded blocking sequence contains two *lox*P sites, between which is a start codon (ATG) followed by a stop codon, creating a short, nonsense open reading frame (ORF). Note that the *lox*P sequence contains two start codons in the same orientation. Therefore, to prevent them from interfering with translation of the coding sequence after *lox*P excision, the *lox*P sites must be orientated such that the ATGs are in the opposite strand.--

Please replace the paragraph on page 30, beginning on line 2, with the following amended paragraph:

-- Methods of the invention which provide for the production of exogenous protein in the avian oviduct and the production of eggs which contain exogenous protein involve an additional step subsequent to providing a suitable vector and introducing the vector into embryonic blastodermal cells so that the vector is integrated into the avian genome. The subsequent step involves deriving a mature transgenic avian from the transgenic blastodermal cells produced in the previous steps. Deriving a mature transgenic avian from the blastodermal cells optionally involves transferring the transgenic blastodermal cells to an embryo and allowing that embryo to develop fully, so that the cells become incorporated into the bird as the embryo is allowed to develop. The resulting chick is then grown to maturity. In an ~~alterantive~~ alternative embodiment, the cells of a blastodermal embryo are transfected or transduced with the vector directly within the embryo. The resulting embryo is allowed to develop and the chick allowed to mature.--

Please replace the paragraph on page 33, beginning on line 3, with the following amended paragraph:

-- Approximately 120 White Leghorns were produced by transduction of the stage X embryos with NLB-CMV-BL transduction particles. These birds constitute chimeric founders, not fully transgenic birds. Extensive analysis of DNA in the blood and sperm from the transduced chickens indicates that 10-20% of the birds had detectable levels of the transgene in any given tissue. Of those birds carrying the transgene, approximately ~~2-15%~~ 0.1 - 1.0% of the cells in any given tissue were actually transgenic. --

Please replace the paragraph on page 34, beginning on line 25, with the following amended paragraph:

-- Significant levels of β-lactamase were detected in the egg white of six hens, as shown in Fig. 4 and Table 1, below. Eggs laid by Hen 1522 ("Betty Lu"), the first hen to demonstrate expression in eggs, have ~~0.3 mg~~ typically have 10 μg or higher of active β-lactamase per egg. Also shown is β-lactamase production from three other NLB-CMV-BL transduced hens (Hen 1549, Hen 1790 and Hen 1593). Every hen that laid eggs containing β-lactamase also had significant levels of β-lactamase in its blood. --

Please replace the table on page 35 with the following amended table:

Table 1. Expression of β–lactamase in eggs of NLB-CMV-BL treated hens.

	Hen #	Average ~~mg~~ μg of β–lactamase per egg	# of eggs assayed
1	Control	~~0.1 ± 0.07~~ -0.49 ± 1.73	~~29~~ 68
2	1522	~~0.31 ± 0.07~~ 9.9 ± 3.1	~~20~~ 59
3	1549	~~0.96 ± 0.15~~ 33.5 ± 7.0	~~22~~ 61

4	1581	~~1.26 ± 0.19~~	69.2 ± 14.9	~~12~~ 51
5	1587	~~1.13 ± 0.13~~	62.6 ± 5.8	~~15~~ 54
6	1790	~~0.68 ± 0.15~~	29.0 ± 2.4	~~13~~ 52
7	1793	~~1.26 ± 0.18~~	49.9 ± 6.2	~~12~~ 51

Control are eggs from untreated hens. The low level of BL in these eggs is due to spontaneous breakdown of PADAC during the course of the kinetic assay. The other hens were transduced with NLB-CMV-BL as described in Example 3. Egg white from each egg was assayed in triplicate.

Please replace the paragraph on page 35, beginning on line 5, with the following amended paragraph:

-- Based on the β-lactamase activity assay, the expression levels of β-lactamase appeared to range from ~~0.1 to 1.3 mg~~ 10 to 70 μg per egg (assuming 40 milliliters of egg white per egg). ~~However, these quantities were significantly lower from the quantities obtained by western blot assay (see Example 5, below) and were determined to be deceptively lower than the true values. The difference in results between the enzymatic activity assay and the western blot analysis (Example 5) was found to be due to the presence of a β-lactamase inhibitor in egg white. The activity of purified β-lactamase was shown to be inhibited by egg white such that 50 ml of egg white in a 200 ml reaction resulted in nearly 100% inhibition, whereas 10 ml of egg white in a 200 ml reaction resulted in only moderate inhibition. Furthermore, spontaneous breakdown of the enzymatic substrate, PADAC, during the course of the assay also contributed to the erroneously low calculation of β-lactamase concentration.~~--

Please replace the paragraph on page 36, beginning on line 5, with the following amended paragraph:

-- Western blot analysis of the same egg white as was assayed in Example 4 confirmed the presence of β-lactamase and provided ~~a more accurate~~ another measurement of the amount of β-lactamase present in the egg than the kinetic assay of Example 4, above.--

Please replace the paragraph on page 36, beginning on line 17, with the following amended paragraph:

-- Various β-lactamase samples were analyzed by western blotting and anti- β-lactamase antibody. The results are shown in Fig. 5. Lanes 1-4 of the blot contain ~~5.2, 1.3, 0.325, and 0.08 µg~~ 236, 59, 15 and 3.6 ng, respectively, of bacterially expressed, purified β-lactamase added to control egg white, forming a standard curve. Lane 5 contains control egg white from an untreated hen. In lane 6 is 2 µl of egg white from Hen 1522 (Betty Lu). Lanes 7-8 contain 1 and 2 µls, respectively, of egg white from Hen 1790. Lanes 9-10 contain 1 and 2 µls, respectively, of egg white from Hen 1793. 1 and 2 µls aliquots of egg white from Hen 1549 was run in lanes 11-12. Lanes 13-14 show 1 and 2 µls, respectively, of egg white from Hen 1581. 2 µls of egg white from Hen 1587 is shown in lane 15.--

Please replace the paragraph on page 37, beginning on line 5, with the following amended paragraph:

-- Based on the western blot results, β-lactamase in lane 6 (from Hen 1522, Betty Lu) is estimated at ~~120 ng, or 2.4 mg per egg~~ 5 ng, or 100 µg per egg, assuming 40 mls of egg white per egg. β-Lactamase in lane 9 (from Hen 1793) is estimated at ~~325 ng~~ 15 ng which corresponds to ~~13 mg~~ 600 µg per egg. The β-lactamase levels per egg as estimated by the western blot analysis were considerably higher (up to 10-fold higher) than the levels estimated by the β-lactamase enzyme assay of Example 4. ~~As explained above, the discrepancy in the protein estimates is believed to be caused by inhibition of enzyme activity by egg white and breakdown~~

~~of the substrate.~~ The discrepancy in the protein estimates is believed to be due to the inherent inaccuracy of the Western blot or enzymatic assays. Alternatively, the amount of protein made is underestimated by the enzymatic assay because only a portion of the oviduct-produced lactamase is active.--

Please replace the paragraph on page 37, beginning on line 13, with the following amended paragraph:

--It should be noted that the ~~up to 13 mg~~ amounts of β-lactamase per egg reported here (up to 600 μg) was produced by chimeric founders, not fully transgenic birds. As reported above, only ~~2-15%~~ 0.1-1.0% of the cells in any given tissue of the chimeric founders were actually transgenic. Assuming that this extent of mosaicism also applies to magnum tissue, then the magnums of the six β-lactamase egg-positive hens were only partially transgenic. Therefore, fully transgenic birds (G_1 offspring) would be expected to express much higher levels, possibly as high as ~~200 mg/egg~~ 20-40 mg/egg. This estimate is significant because it indicates that non-magnum specific promoters such as CMV can effectively compete with magnum specific genes such as *ovalbumin* and *lysozyme* for the egg-white protein synthesis machinery.--

Please replace the paragraph on page 38, beginning on line 6, with the following amended paragraph:

-- Cells are dispersed by brief trypsin (0.2%) digestion, washed once by low speed centrifugation in Dulbecco's modified Eagle's medium (DMEM) and then transfected with linearized plasmids via lipofectin (~~16 mg/200 ml~~ 16 μg/200μg, BRL) for 3 hours at room temperature. The vectors shown in Figures 1, 3, or 4 would serve as suitable expression constructs here. Cells are washed free of lipofectin with medium and then 400-600 cells are injected into ~~g-irradiated~~ γ-irradiated (650 rads) recipient stage X embryos from the Athens-Canadian randombred line (AC line). Injection is through a small window (~0.5 cm) into the subgerminal cavity beneath the recipient blastoderms. Windows are sealed with fresh egg shell membrane and Duco plastic cement. Eggs are then incubated at 39.1°C in a humidified incubator with 90° rotation every 2 hr.--

Please replace the paragraph on page 39, beginning on line 10, with the following amended paragraph:

-- The isolated DNA is then tested for the presence of the transgenes using the Taqman sequence detection assay to evaluate the efficiency of the embryo transduction process. The Taqman sequence detection system allows the direct detection of a specific sequence. A fluorescently-labeled oligonucleotide probe complementary to an internal region of a desired PCR product only fluoresces when annealed to the desired PCR product, which in this case is complementary to the transgene. Because all of the detection occurs in the PCR tube during the cycling process, the Taqman system allows high-throughput PCR (no gel electrophoresis is need) as well as sequence detection analogous to and as sensitive as Southern analysis. 1 μl of the isolated DNA, which contains 600-800 ng of DNA, is used for the Taqman reaction. Each reaction contains two sets of primer pairs and Taqman probes. The first set detects the chicken glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) and is used as an internal control for the quality of the genomic DNA and also serves as a standard for quantitation of the transgene dosage. The second set is specific for the desired transgene. ~~Fluorescence is detected in a dissecting stereomicroscope equipped with epifluorescence detection.~~ The two Taqman probes are attached to different dyes which fluoresce at unique wavelengths: thus both PCR products are detected simultaneously in an ABI/PE 7700 Sequence Detector. It is estimated that up to 180 birds will hatch, and 20% (36 birds) will contain the transgene in their blood.--

Please replace the paragraph on page 40, beginning on line 23, with the following amended paragraph:

-- Following transfection with a PMGI targeting vector like that of ~~Figure 4~~ <u>Figure 9</u>, cells are grown for one day in the absence of a feeder layer and green cells separated from blue/green cells using a fluorescence-activated cell sorter the next day. Green cells are then briefly passaged on feeder cells prior to injection into recipient embryos. Green cells are also screened as above for correct insertion.--